II. REMARKS

Upon entry of the amendment and new claims, claims 1-18 and 27-33 will be pending. Claims 4-5, and 17-18 have been withdrawn.

A. Regarding the Amendment

Claim 15 has been amended to clarify that the "tumor cells comprise tumor cells" of a tissue as recited. The amendment merely addresses an informality, wherein the previously recited term "cell proliferative disorder" lacked antecedent basis, and, therefore, does not add new matter.

New claims 27 to 33 have been added. The new claims are supported by the claims as originally filed. For example, new claim 27 is supported by original claims 1, 3, and 4; new claims 28 and 29 are supported by original claims 2 and 5, respectively; new claim 30 is supported by original claims 6, 8, and 10; and new claims 31 to 33 are supported by original claims 12, 13, and 14, respectively. As such, new claims 27 to 33 do not add new matter.

B. Regarding the Restriction Requirement

The election of claims 1-3, and 6-16, wherein claims 1 and 6-16 are generic linking claims, is acknowledged. As indicated in the Office Action, upon determining that the linking claims are allowable, the restriction requirement will be withdrawn with respect to the linked inventions, and any claims depending from or otherwise including all the limitations of the allowable linking claims will be entitled to examination in the instant application. As such, claims withdrawn pursuant to the restriction requirement remain pending.

It is noted that the Office Action inadvertently identifies claims 18-19 as withdrawn. It is submitted that claims 17 and 18 properly should be indicated as withdrawn; claims 19 to 26 have been cancelled. It is further noted that newly added claim 29 is directed to the non-elected subject matter and, therefore, should be considered withdrawn pending a determination that the claims are otherwise in condition for allowance.

C. Rejections under 35 U.S.C. § 112

The objection to the specification and corresponding rejection of claims 1-3 and 6-16 under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement are respectfully traversed.

It is acknowledged in the Office Action that the specification is enabling for a method of inhibiting AS-30D hepatoma cells in culture via expression of SEQ ID NO:1, which encodes Type II hexokinase, in an antisense orientation. It is alleged, however, that the specification fails to disclose how to treat the vast array of cancers encompassed within the claims, that no correlation is provided to show a correlation of hexokinase overexpression as causative of the broad range of cancers, and that no particular target for a particular disease is provided. As such, it is alleged that undue experimentation would have been required for one skilled in the art to practice the claimed methods.

Although it is alleged that the breadth of cancers contemplated for treatment according to the claimed methods is vast, Applicants point out that the cancers amenable to treatment according to a method of the invention share the common characteristic of having a highly glycolytic phenotype due to hexokinase activity. As such, while a variety of different cancers can be treated according to the claimed methods, the skilled artisan would have known that all such

cancers are related in comprising tumor cells having a highly glycolytic phenotype due to hexokinase activity.

Further in this respect, the specification discloses that hexokinase is expressed in a variety of different tissue types, and lists the major tissue locations for various hexokinases, including brain, kidney, muscle and adipose tissue (see Table 1, page 9; see, also, page 9, line 21, to page 10, line 3). In addition, the specification discloses that hexokinase is markedly elevated in highly glycolytic, rapidly growing tumors (page 9, lines 3-14), and discloses methods of confirming that a tumor cell has a highly glycolytic phenotype due to hexokinase overexpression and, therefore, is suitable for treatment according to the claimed methods (see, for example, page 8, lines 11-15, and page 38, line 20, to page 39, line 10). Further, a method of the invention is exemplified using hepatoma cells, which have a highly glycolytic phenotype and the proliferation of which can be inhibited using antisense molecules that can inhibit the expression of Type I and Type II hexokinases (see Example 4, at pages 40-41).

In summary, tumor cells amenable to treatment according to a method of the invention, while potentially originating from any of a variety of different tissue types, share the common feature of a highly glycolytic phenotype due to hexokinase activity. As such, it is submitted that undue experimentation would not have been required for one skilled in the art to practice the claimed methods because the specification exemplifies the effectiveness of a method of the invention to inhibit proliferation of such tumor cells, and because methods for confirming that a tumor cell has such a highly glycolytic phenotype are well known and exemplified in the specification. Accordingly, removal of the rejection as it pertains to the scope of tumor cells to be treated is respectfully requested.

It is further alleged that inhibition of tumor cell proliferation by inhibiting Type I and/or Type II hexokinase in one cell line does not correlate with inhibition of tumor cell growth in other cells. Applicants are uncertain as to the basis of this rejection, and submit that there is no reason to believe that the exemplified method of inhibiting tumor cell proliferation via antisense inhibition of hexokinase in AS-30D hepatoma cells would not similarly be effective with respect to any other tumor cells having a highly glycolytic phenotype due to hexokinase activity.

In support of this position, Applicants point out that the claimed methods are analogous to the methods described in Exhibits A and B (submitted herewith), wherein a hexokinase inhibiting compound, 3-bromopyruvate, was demonstrated to inhibit the proliferation of various tumor cell types having a highly glycolytic phenotype. For example, Ko et. al. (Cancer Lett. 173:83-91, 2001, a copy of which is attached as Exhibit A) reported that 3-bromopyruvate (3BrPA) inhibited proliferation of the highly glycolytic AS-30D hepatoma tumor cells (see, for example, page 90, section 3.4), which are the same hepatoma cells exemplified in the current specification. Geschwind et. al. (Cancer Res. 62:3909-3913, 2002, a copy of which is attached as Exhibit B), using an animal model, reported that 3BrPA also inhibited proliferation of liver-implanted VX2 epidermoid tumor cells, which have a highly glycolytic phenotype, as well as secondary tumors, including highly glycolytic tumors that had developed in the lungs (see Exhibit B, Abstract; see, also, page 3913, left column, first full paragraph). Correspondingly, 3BrPA also inhibited glycolysis in VX2 tumor cells (see Exhibit A, Figure 3B at page 88, and page 89, right column, first full paragraph).

As such, Ko et al. (Exhibit A) demonstrate that 3BrPA effectively inhibited the proliferation of AS-30D hepatoma cells, which exhibit a highly glycolytic phenotype, and Geschwind et al. (Exhibit B) extend the results of Ko et al. to tumor cells of a non-hepatic origin by demonstrating that 3BrPA also inhibited the proliferation of VX2 epidermoid tumor cells,

which exhibit a highly glycolytic phenotype. Thus, the results reported in Exhibits A and B confirm that, as disclosed in the subject application, an agent that suppresses the highly glycolytic phenotype of a tumor cell, including a hepatoma and an epidermoid tumor, can inhibit proliferation of the tumor cells. As such, it is submitted that Exhibits A and B, which includes work of the inventors of the subject application, provide confirmatory evidence that undue experimentation would not have been required for one skilled in the art to inhibit proliferation of various types of tumor cells having a highly glycolytic phenotype, including cells in a whole animal.

It is also alleged that the claimed methods lack enablement because the specification has fails to show a correlation that hexokinase overexpression is causative of a broad range of cancers. The Office Action cites to Newgard et al. (U.S. Pat. No. 5,891,717, column 17) indicating that a correlation of hexokinase activity and cell transformation has not been proven to exist as a cause and effect relationship. Applicants point out, however, for clarification, that Newgard et al. go on to state that "the reduction of hexokinase activity in a cell line by any suitable method...is contemplated to be of use in inhibiting cell growth" (col. 17, lines 51-54). As such, Newgard et al. appear to acknowledge a correlation between hexokinase activity and cell proliferation.

Regardless of the teaching of Newgard et al., however, Applicants submit that it is not relevant to the claimed methods whether hexokinase activity is causative of a cancer, or whether there is any cause and effect relationship of hexokinase expression and cell transformation. It has long been established that there is no requirement that an inventor understand the mechanism by which an invention works in order to meet the enablement requirement:

> ...it is axiomatic that an inventor need not comprehend the scientific principles on which the practical effectiveness of his invention rests, nor is the inventor's theory or belief as to how his invention works a necessary element in the specification to satisfy the enablement requirement of 35 U.S.C. § 112. [Citation omitted].

Cross v. Iizuka 224 U.S.P.Q 739, 741, note 3 (Fed. Cir. 1985)). In the present case, the specification discloses that an antisense hexokinase molecule reduces hexokinase activity by as much as 93% in tumor cells having a highly glycolytic phenotype due to hexokinase activity (page 40, lines 20-23), and that decreased cell proliferation correlated with the decreased hexokinase activity (page 40, lines 24-26; page 41, lines 1-3; and Figures 4 and 5). Further, in view of the expression of hexokinases in various tissues and of the identification of a highly glycolytic phenotype due to hexokinase activity in various types of tumor cells, there is no reason to suspect that the claimed methods would not be equally effective in reducing the proliferation of highly glycolytic tumor cells other than hepatoma cells, a position that is supported by the results of Ko et al. (Exhibit A) and of Geschwind et al. (Exhibit B). Thus, in view of the effectiveness of a method of the invention as exemplified using hepatoma cells, it is submitted that it is not relevant whether there is a cause and effect relationship between hexokinase activity and cancer and, therefore, it is respectfully requested that this basis for the rejection be removed.

It is further alleged that the specification has failed to demonstrate that the inhibition of cell growth is due to inhibition of either a Type I or Type II hexokinase alone. As above, it appears that this basis of the rejection is based on a requirement of a disclosure of the mechanism by which the claimed methods work. Applicants respectfully submit, however, that it is irrelevant whether the exemplified inhibition of proliferation of highly glycolytic tumor cells was due to inhibition of Type I hexokinase or Type II hexokinase or both.

Notwithstanding Applicants position that it is not relevant whether Type I hexokinase or Type II hexokinase or both were inhibited by the antisense Type II hexokinase molecule, it is noted that the close homology of the Type I and Type II hexokinase isotypes is recognized in the specification (see, for example, page 11, line 13, to page 12, line 11) and well known in the art (see Applicants' previous response mailed May 12, 2003, at page 7, first paragraph, setting forth the substantial sequence identity shared among SEQ ID NO:1 and SEQ ID NO:2; see, also, Exhibits A and B attached thereto), and the specification discloses that it is "highly likely" that the antisense Type II hexokinase molecule used in the exemplified methods also targeted the Type I hexokinase mRNA (paragraph bridging pages 40 to 41). As such, Applicants submit that, in view of the specification, one skilled in the art reasonably would have known that, due to the substantial sequence identity shared among the polynucleotides encoding Type I and Type II hexokinases, that an antisense molecule based on either hexokinase likely would inhibit the activity of both hexokinases, if expressed, in a tumor cell having a highly glycolytic phenotype due to hexokinase activity and, therefore, would inhibit proliferation of the tumor cells, as claimed. Accordingly, it is respectfully requested that this basis of the rejection be removed.

In summary, the specification discloses that an antisense hexokinase molecule can inhibit cell proliferation of tumor cells having a highly glycolytic phenotype due to hexokinase activity, and exemplifies the claimed methods using hepatoma cells having such a highly glycolytic phenotype. As such, it is submitted that one skilled in the art, viewing the subject application, would have known how to practice the claimed methods without undue experimentation, and further submitted that the Ko et al. (Exhibit A) and Geschwind et al. (Exhibit B) references provide confirmatory evidence that a method of the invention reasonably can be expected to be effective in any tumor cells having the required phenotype. Accordingly, it is respectfully requested that the Examiner reconsider and remove the rejection of the claims under 35 U.S.C. 112, first paragraph.

The rejection of claim 15 under 35 U.S.C. 112, second paragraph, as allegedly being indefinite is respectfully traversed.

The Examiner points out that the term "said proliferative disorder" lacks sufficient antecedent basis. The claim has been amended to clarify the subject matter regarded as the invention. As such, it is respectfully requested that this rejection be removed.

In view of the amendments and the above remarks, it is submitted that the claims are in condition for allowance, and a notice to that effect is respectfully requested. The Examiner is invited to contact Applicants' undersigned representative if there are any questions relating to this application.

Please charge any additional fees, or make any credits, to Deposit Account No. 50-1355.

Respectfully submitted,

Date: October 17, 2003

Lisa A. Haile, J.D., Ph.D.
Reg. No. 38,347
Attorney for Applicants
Telephone: (858) 677-1456
Facsimile: (858) 677-1465

USPTO CUSTOMER NUMBER 28213
GRAY CARY WARE & FREIDENRICH LLP
4365 Executive Drive, Suite 1100
San Diego, CA 92121-2133

Enclosures: Exhibits A and B

EXHIBIT A

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Glucose catabolism in the rabbit VX2 tumor model for liver cancer: characterization and targeting hexokinase

Young Hee Ko[a], Peter L. Pedersen[a,*], J.F. Geschwind[b]

[a]*Department of Biological Chemistry, Johns Hopkins University, School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205-2185, USA*
[b]*Division of Cardiovascular and Interventional Radiology, Johns Hopkins University, School of Medicine, Baltimore, MD, USA*

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Abstract

The rabbit VX2 tumor when implanted in the liver has proven convenient as a model for studying hepatocellular carcinomas. However, its metabolic properties have not been well studied. Significantly, studies described here show that the VX2 tumor exhibits a high glycolytic/high hexokinase phenotype that is retained following implantation and growth in rabbit liver. In addition, results of a limited screen show that the glycolytic rate is inhibited best by 2-deoxyglucose (2DOG) and 3-bromopyruvate (3BrPA), the former compound of which is phosphorylated by hexokinase but not further metabolized, while the latter directly inhibits hexokinase. Finally, when tested on hepatoma cells in culture both inhibitors facilitated cell death. These studies underscore the usefulness of the VX2 tumor model for the study of advanced liver cancer and for selecting anti-hepatoma agents. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Liver cancer; Tumor metabolism; Hexokinase; Drug targeting; 2-Deoxy Glucose; 3-Bromo Pyruvate

1. Introduction

Liver cancer, in particular hepatocellular carcinoma (hepatoma) remains one of the most common fatal cancers in the world [1]. The clinical prognosis is very poor with the medium survival time approaching 6 months. In recent years the VX2 tumor, an epidermoid rabbit tumor induced by the Shope papilloma virus [2], has shown promise as a model system for studying liver cancer [3,4]. This is because it grows well when implanted in the rabbit's liver where it takes on growth properties and a vascularization system similar to many human liver tumors [3,4]. Here, the tumor is fed through a hepatic artery while the liver is fed through the portal vein. Thus, it is possible via the method known as transcatheter chemoembolization [5,6] to deliver anticancer agents directly to the tumor via the hepatic artery [5,6]. In addition, it has been shown that when delivery is made using certain oils the mixture preferentially localizes in the tumor rather than in the surrounding liver tissue [3,4]. This is important as it may allow for the targeting of exceptionally potent cancer killing agents directly to the tumor for brief periods of time thus minimizing damage to the surrounding liver tissue and toxicity to the host.

With the above in mind, it is important to learn more about the energy metabolism of the VX2 tumor in order to determine to what extent it mimics that of rapidly growing hepatomas. These cancers are known to exhibit a high glucose catabolic rate [7,8],

* Corresponding author. Tel.: +1-410-955-3827; fax: +1-410-614-1944.
E-mail address: ppederse@welchlink.welch.jhu.edu (P.L. Pedersen).

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and where examined carefully, to contain elevated levels of hexokinase bound to their mitochondria [9–11]. Moreover, in the AS-30D hepatoma, the most extensively studied tumor in this class, it has been shown also that the gene for hexokinase is amplified [12] and that the mRNA levels are markedly elevated [13]. In experiments described below, we demonstrate clearly that the VX2 tumor does in fact exhibit a high glycolytic/high hexokinase phenotype, and that a large fraction of the total cell hexokinase is mitochondrially bound. In addition, we report the results of a limited screen for inhibitors of VX2 tumor glycolysis that may prove useful in future chemoembolization experiments designed to specifically target these agents to the tumor growing in the host liver, and, once there, arrest tumor cell growth.

2. Materials and methods

2.1. Materials

New Zealand white rabbits weighing 3.5–4.2 kg were obtained from Robinson Services Inc. The VX2 tumor, normally grown in the hind limb of these animals, was obtained locally from Dr John Hilton, Department of Oncology, Johns Hopkins University School of Medicine. AS-30D hepatoma cells, an established line, is maintained by Min Gyu Lee in the authors' laboratory. This is done by growth and passage of the cells in the peritoneal cavity of female Sprague–Dawley rats (Charles River Breeding Laboratories). The following agents were obtained from Sigma: D-glucose, 2-deoxyglucose, 2-fluoro-2-deoxyglucose, 6-fluoro-6-deoxyglucose, 3-O-methyl-glucose, 5-thio-D-glucose-6-phosphate, L-glucose, D-xylose, D-lyxose, 3-bromopyruvic acid, ATP, ADP, $NADP^+$, D-mannitol, Hepes, succinate, oligomycin, and bovine albumin. The lactic acid kit containing lactic dehydrogenase, NAD^+, hydrazine, and a glycine buffer, pH 9.2 was obtained also from Sigma. NaP_i, KP_i, and sucrose were obtained from J.T. Baker, and the Coomassie dye binding agent from Pierce. Glucose-6-phosphate dehydrogenase was obtained from Roche Molecular Biochemicals, and both the DMEM tissue culture medium and trypan blue were from Life Technologies Gibco BRL. The Clark oxygen electrode was purchased from Yellow Springs Instruments.

2.2. Processing the VX2 tumor for biochemical analyses

In one set of studies the VX2 tumors, which had grown in the hind limb of New Zealand white rabbits for 4 weeks, were excised, cut into 1 g pieces (ca. $10 \times 10 \times 10$ mm) with a razor blade, and then subjected to assays described below for monitoring both glycolytic and hexokinase activities. In a second set of studies VX2 tumors, which had grown in the hind limb of a New Zealand white rabbit for about 2 weeks, were excised, broken into small chunks (<0.1 g), and then implanted into the livers of a number of other rabbits. Following implantation, VX2 tumors rapidly developed in the livers of each animal. They were excised at different times ranging from 2 to 5.7 weeks and also subjected to the assays described below for monitoring glycolytic and hexokinase activities, as well as an assay for monitoring mitochondrial respiration. In all cases the exterior surface of the tumor was shaved to remove any remaining normal tissue. In addition, special care was taken to assure that only the viable portion of the tumor located near or on the surface was removed for analyses. The preparation of the animals for surgery, and the surgical and implantation procedures, have been described in detail elsewhere [4]. These procedures were approved by the Animal Care and Use Committee at the Johns Hopkins University School of Medicine and conducted according to their guidelines.

2.3. Assay for glycolytic activity

Glycolysis was assayed by monitoring the formation of lactic acid following the addition of glucose to a medium containing VX2 tumor slices. Specifically, freshly excised tumors were washed 3 times at 4°C in 20 ml Chance Hess Medium containing 6.2 mM KCl, 154 mM NaCl, and 11 mM NaP_i, pH 7.4. Slices, 1 g each, were then prior incubated for 30 min in a Lab-Line incubator-shaker at 37°C in 2 ml of the same medium while shaking at 50 rev/min. Glucose was then added to give a final concentration of 6 mM, after which the incubation/shaking process was continued with 0.2 ml samples being removed every 30 min for up to 2 h. Then, samples were removed for

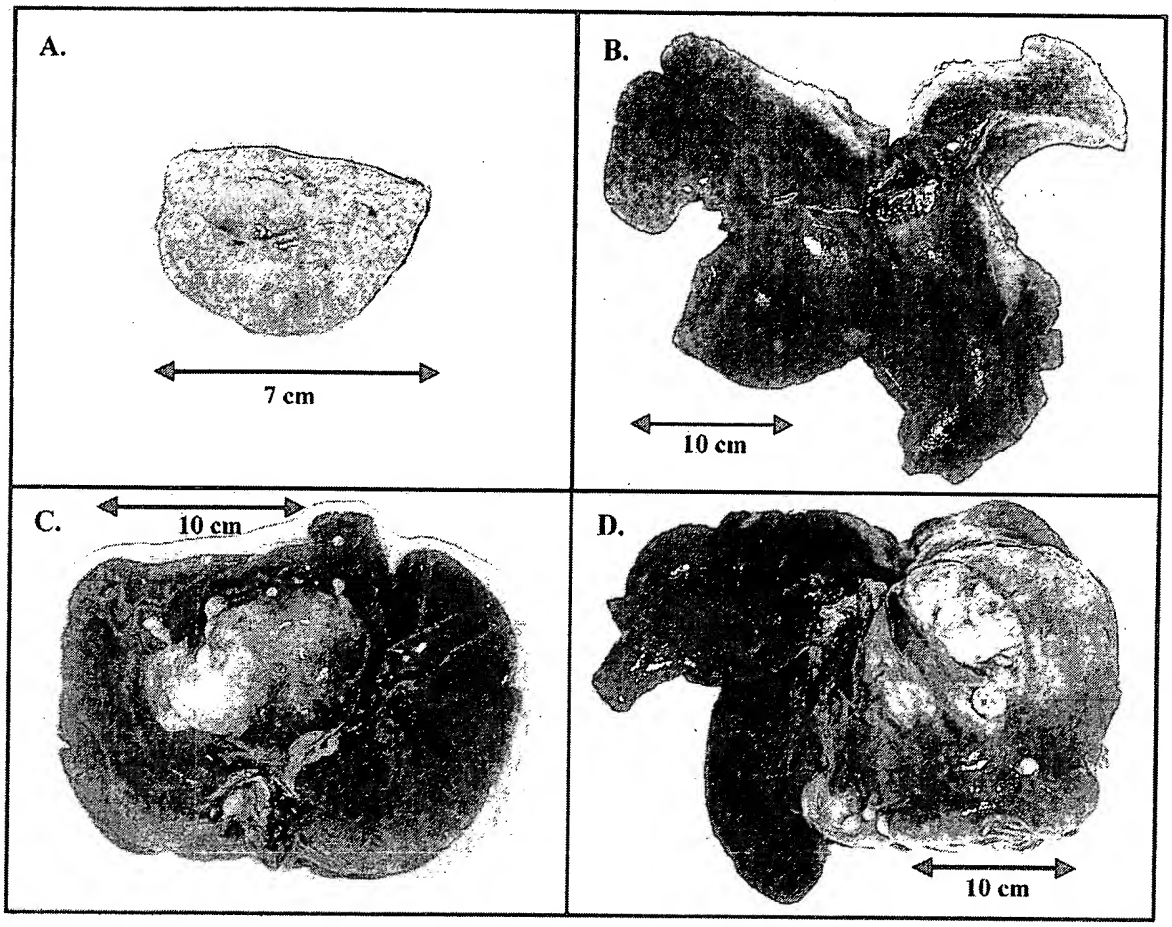

Fig. 1. Photographs of the VX2 tumor. (A) An excised VX2 tumor after 4 weeks growth in the rabbit hind limb. (B) Control liver isolated from a rabbit of the same age. (C,D) Livers harboring, respectively, VX2 tumors after 4 and 5.7 weeks of implantation.

analysis at two additional time points indicated Fig. 2. These samples were subjected to centrifugation at 14,000 rev/min in an Eppendorf Centrifuge (Model 5415C). Then, aliquots of the supernatant, 0.01 ml, were removed from each sample, diluted with 0.03 ml of water and subjected to lactic acid determination using the kit supplied by Sigma. The latter contains, in addition to lactic dehydrogenase and NAD^+, hydrazine, and glycine buffer, pH 9.2. The mixture was incubated for 30 min at 25°C after which the absorbance due to formation of NADH was determined at 340 nm using a Gilford Model 260 spectrophotometer. To assess the elevation of the glycolytic activity of the VX2 tumor over that of liver tissue, parallel studies were always carried out in an identical manner with 1 g liver slices derived from the host liver. Finally, in those cases where agents were tested for their capacity to inhibit glycolysis, these were introduced into the system at the prior incubation step with all subsequent steps being run in parallel with those described above for the VX2 tumor or liver alone.

2.4. Preparation of subcellular fractions for hexokinase and mitochondrial respiration assays

The freshly isolated liver or VX2 tumor tissues (15–20 g wet weight) was rinsed in three volumes of H-medium (210 mM D-mannitol, 70 mM sucrose, 2 mM Hepes, and 0.05% bovine albumin, pH 7.4) at 4°C and minced with a razor blade as finely as possi-

ble. A 30% (V/V) suspension was made using ice-cold H-medium in a Potter–Elvehjem glass homogenizer (55 ml capacity), and homogenization was achieved by applying four complete up and down strokes through the suspension with a rotating (~400 rpm), serrated, Teflon pestle attached to a motor. To remove cell debris and nuclei, the resultant homogenate was diluted to twice the initial volume and centrifuged at $630 \times g$ for 8 min at 4°C in a Sorvall RC-2B centrifuge using a GSA rotor. The supernatant was removed and then centrifuged at $6800 \times g$ for 15 min under the same conditions. The resultant supernatant was saved and referred to here as the cytosolic faction. The pellet was resuspended in the initial volume of H-medium and centrifuged twice at $9800 \times g$ for 15 min at 4°C. The washed pellet represents the mitochondrial fraction.

2.5. Assay for hexokinase activity

The assay coupled the glucose-6-phosphate formed in the hexokinase reaction to the glucose-6-phosphate dehydrogenase reaction [10]. Here, $NADP^+$, oxidizes glucose-6-phosphate to a γ-lactone while becoming reduced to NADPH, thus allowing the formation of the latter to be monitored spectrophotometrically at 340 nm. The final reaction mixture contained the following ingredients in a total volume of 1 ml at 25°C: 25 mM triethanolamine, pH 7.6, 15 mM $MgCl_2$, 1 mM dithiothreitol, 0.45 mM NaCN, 0.005 mg/ml oligomycin, 0.014 mM DAPP [P^1,P^5-Di (adenosine-5′) pentaphosphate], 5 mM ATP, 3.3 units glucose-6-phosphate dehydrogenase, 1 mM $NADP^+$, 0.1–0.3 mg cytosolic or mitochondrial fraction, and concentrations of glucose as indicated in Figs. 1–4. Glucose was used to initiate the reaction.

2.6. Assay for mitochondrial respiration

Oxygen consumption rates were measured polarographically using a Clark oxygen electrode inserted into a 2.5 ml chamber equipped with a magnetic stirrer. The electrode was connected to a chart recorder calibrated between 0 and 100% saturation with atmospheric oxygen at 25°C. The loss of oxygen was monitored in a 2.1 ml system at 25°C containing 1 mg mitochondria, 0.5 mM EDTA, 2.0 mM Hepes, 220 mM D-mannitol, 70 mM sucrose, 2.5 mM KP_i, 2.6 mM $MgCl_2$, and 0.5 mg/ml bovine albumin, and, where indicated, 7.8 mM succinate (respiratory substrate), and 0.24 mM ADP (ATP synthesis substrate).

2.7. AS-30D hepatoma cells: culture conditions, and assessment of cell viability

AS-30D hepatoma cells grown in the peritoneal cavity of Sprague–Dawley female rats (see Section 2.1) were adapted to grow in tissue culture in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2, and counted in a Neubauer chamber after trypan blue dye addition (cell/dye volume = 1/1) by visualization under a Nikon inverted microscope.

2.8. Protein determination

Protein was determined using Pierce's Coomassie dye binding assay protocol.

3. Results

3.1. Description of the VX2 tumor prior to and after liver implantation

Photographs of VX2 tumors representative of those used in this study are presented in Fig. 1. When excised from the hind limb of the New Zealand white rabbit (donor) at 4 weeks the tumor is about 10 g in weight, flesh colored with some surface vascularization, and without any obvious signs of necrosis (Fig. 1A). When small chunks (<0.1 g) are implanted in the liver of a rabbit of similar size and age the tumor grows rapidly attaining a weight as high as 25 g in a 4 week period while retaining its solid, flesh colored features (Fig. 1C). Here too, there are no obvious signs of necrosis although an increased surface vascularization is apparent. At 5.7 weeks (Fig. 1D), the liver implanted VX2 tumor has become highly vascularized on its surface and more than doubled in size in the intervening 1.7 weeks. Much of the increased weight is due to fluid that has accumulated within the core of the tumor where it has become almost completely necrotic and taken on a mush-like texture. At this stage only the tissue near the surface of the

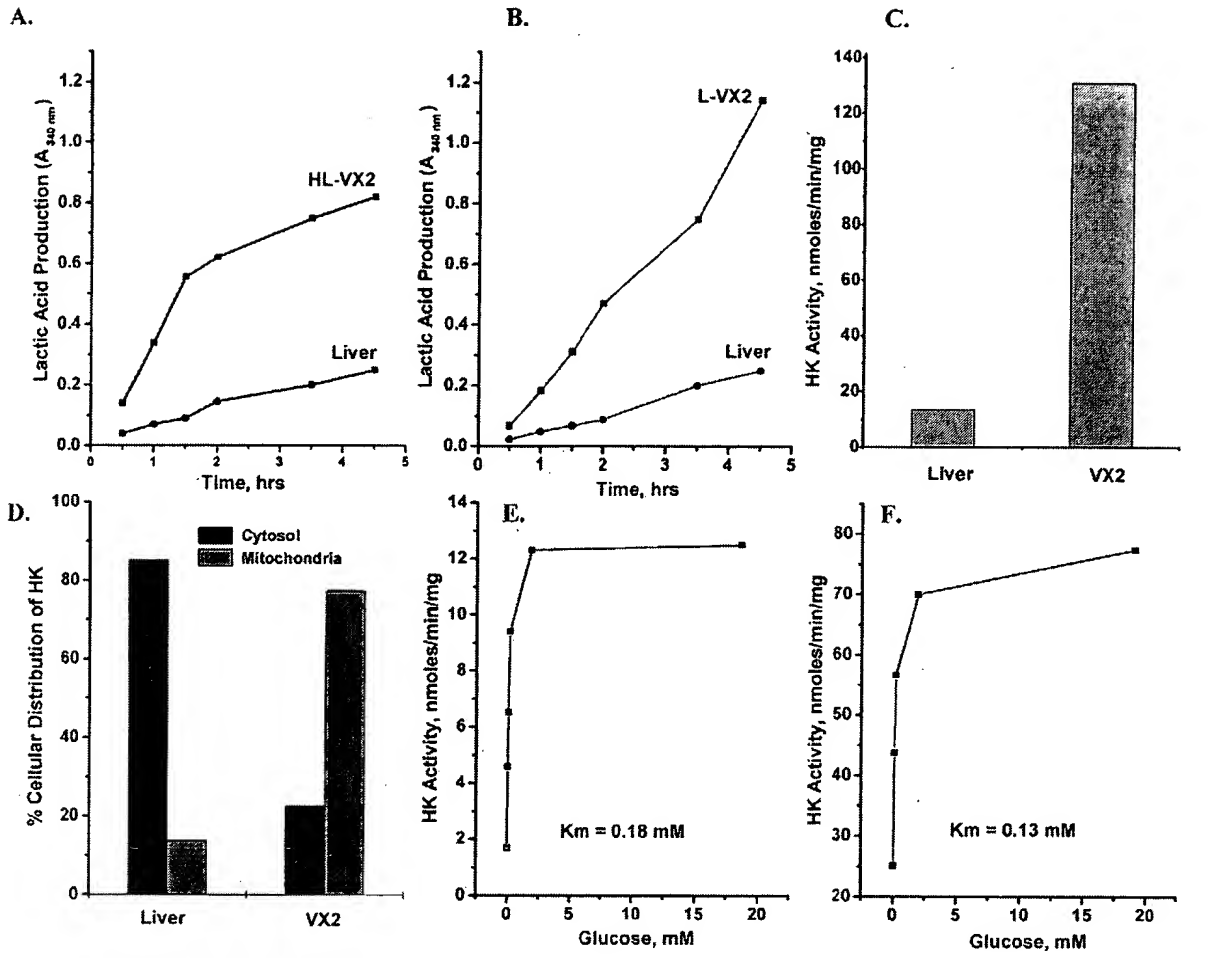

Fig. 2. (A,B), Lactic acid production by the VX2 tumors isolated, respectively, from the rabbit hind limb (HL-VX2) and rabbit liver (L-VX2). In both cases, slices of the tumors were incubated in the presence of 6 mM glucose for the times indicated and then assayed for lactic acid exactly as described in Section 2. Liver slices from the animals from which the tumors were obtained were subjected to the same assay. (C) Comparison of the activity of hexokinase per mg protein in liver and VX2 tumor tissues. The mean ± standard error are 13.7 ± 2.5 (liver) and 131 ± 10.1 (tumor). See Section 2 for assay. (D) Relative distribution of total hexokinase activity in the mitochondrial and cytosolic fractions of rabbit liver and VX2 tumor tissues. For liver, the mean ± standard error in % of total cellular distribution is 85.3 ± 2.6 (cytosol) and 13.7 ± 2.5 (mitochondria). For the VX2 tumor these values are 22.6 ± 6 (cytosol) and 77.4 ± 6 (mitochondria). The % of the total starting protein recovered in the mitochondrial fraction (~20–21%) was nearly the same for liver and tumor. (E,F) Michaelis–Menten kinetic constants (Km values for glucose) for hexokinase located, respectively, in the cytosolic and mitochondrial fractions of the VX2 tumor. The mean ± standard error is 0.18 ± 0.015 mM (cytosolic fraction) and 0.13 ± 0.02 mM (mitochondrial fraction).

tumor remains viable and can be used for biochemical studies. Tumors were not carried beyond 5.7 weeks.

3.2. Glycolytic capacity and hexokinase activity of the VX2 tumor prior to and after liver implantation

Results of experiments presented in Fig. 2A show that slices of the VX2 tumor isolated after 4 weeks of growth in the hind limb of a New Zealand white rabbit exhibit a glycolytic rate that is 7.4 fold higher than that observed for normal liver tissue derived from the same animal. Significantly, this enhanced glycolytic rate of the VX2 tumor relative to liver tissue is retained when small chunks of the tumor (<0.1 g)

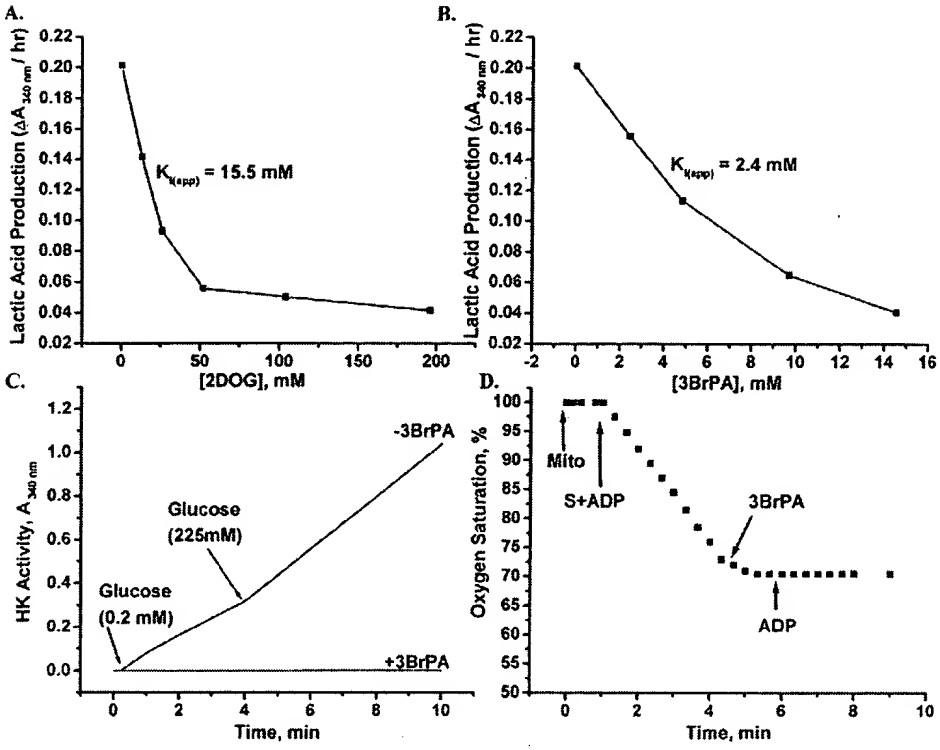

Fig. 3. (A,B) Comparison of the inhibitory effects, respectively, of 2DOG and 3BrPA on the glycolytic capacity of the isolated liver implanted VX2 tumor. In both cases various concentrations of 2DOG and 3BrPA were prior incubated for 30 min at 37°C with 1 g VX2 tumor slices. Glycolysis was monitored exactly as described in Section 2. Under the assay conditions used, 3BrPA was without effect on the activity of lactate dehydrogenase. C, The effect of 3BrPA on the activity of hexokinase bound to the mitochondrial fraction of the VX2 tumor. Hexokinase activity was monitored exactly as described in Section 2 in the presence and absence of 5 mM 3BrPA. Under the assay conditions used, 3BrPA was without effect on the activity of glucose-6-phosphate dehydrogenase. (D), The effect of 3BrPA on the ADP stimulated respiratory rate of rabbit VX2 tumor mitochondria. The procedures for preparing the mitochondria and assaying respiration are described in Section 2. Where indicated, 3BrPA was added to give a final concentration of 1.2 mM.

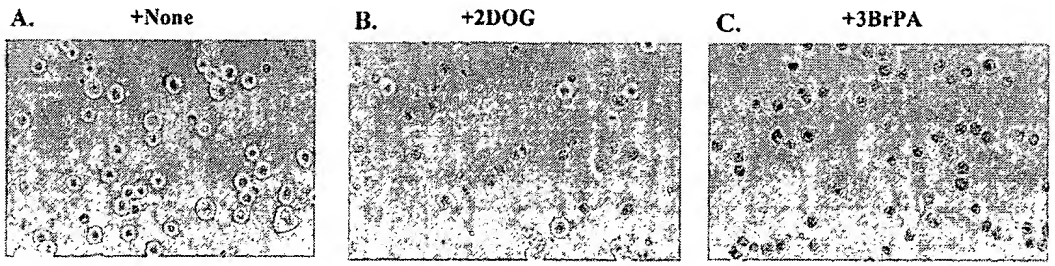

Fig. 4. Comparison of the effects of 2DOG and 3BrPA on the viability of rat hepatoma cells growing in tissue culture. Cells were maintained in tissue culture exactly as described in Section 2. Where indicated, 20 mM 2DOG (B) or 5 mM 3BrPA (C) were added to cells that had been growing for 24 h. After an additional 12 h, the viability of the cells was assessed as described in Section 2 using trypan blue. In (A) (Control) almost all cells in the field are viable as indicated by a dark nucleus and a surrounding bright appearing cytosol. The remaining viable cells in (B) (2DOG) showing this appearance are indicated by arrows. In (C) (3BrPA) there are no such cells as they have all taken up the trypan blue.

are implanted in the liver of another rabbit of similar size and age. Thus, Fig. 2B shows that the glycolytic rate of tumor slices derived from the liver implanted VX2 tumor after 4 weeks of growth is 8.3 fold higher than the glycolytic rate obtained with liver slices obtained from the same liver in which the tumor implant had been made. In data not presented here, this high glycolytic rate of the liver implanted VX2 tumor remained relatively constant through 5.7 weeks of growth.

Results presented in Fig. 2C show that the hexokinase activity is also markedly elevated (~ 9.5 fold) in the liver implanted VX2 tumor relative to the activity of this enzyme in the surrounding liver tissue. In addition, as shown in Fig. 2D, the subcellular distribution of this activity in the tumor (~70% in the mitochondrial fraction and ~30% in the cytosol) differs markedly from that in the surrounding liver tissue (~20% in the mitochondrial fraction and 80% in the cytosol). Finally, Fig. 2E,F show that the Km of the tumor hexokinase(s) for glucose is very low (0.11 mM, mitochondrial fraction and 0.19 mM, cytosolic fraction, mean value of three experiments) reflecting a high apparent affinity of the isoform(s) for glucose. This is in sharp contrast to liver where the Km for glucose of the major hexokinase isoform involved, i.e., glucokinase, is at least 5 mM in most reported studies [14].

These studies show clearly that the VX2 tumor, although of non-hepatic origin, exhibits glucose metabolic properties characteristic of many rapidly growing hepatomas [7–11], the biochemical hallmarks of which are a high glycolytic/high hexokinase phenotype, and binding of the hexokinase (low Km for glucose) to the mitochondrial fraction.

3.3. Identification of inhibitors of the glycolytic capacity of the isolated liver implanted VX2 tumor

A limited screen was carried out to identify inhibitors of the glycolytic capacity of the VX2 tumor with the purpose of selecting agents that might prove effective in arresting tumor cell growth. The screen included the following nine compounds: 2-deoxyglucose (2DOG), 2-fluoro-2-deoxyglucose, 6-fluoro-6-deoxyglucose, 3-*O*-methyl glucose, 5-thio-D-glucose-6-phosphate, L-glucose, D-xylose, D-lyxose, and 3-bromopyruvic acid (3BrPA). The screen was conducted by incubating VX2 tumor slices in a medium containing 6 mM glucose with 6 mM of each of these agents at 37°C for 5 h. The use of 6 mM glucose in the medium was used to mimic the maximal amount of glucose that might be in the blood in a real in vivo situation. Interestingly, in data not presented here, all glucose analogs or other sugars tested under these conditions proved to be ineffective as inhibitors of VX2 tumor glycolysis indicating the inability of these sugars to act as effective glycolytic inhibitors under the conditions specified. In sharp contrast, the pyruvate analog (3BrPA) almost completely inhibited glycolysis.

With the above preliminary data at hand, only two of the nine compounds screened were studied in more detail. One was 2DOG because under certain conditions it is known to inhibit glycolysis when it is phosphorylated by hexokinase to 2DOG-6-P which cannot be further metabolized [15]. The other compound tested in more detail was 3BrPA because of its effectiveness as a glycolytic inhibitor in the preliminary screen. Data presented in Fig. 3A show that 2DOG can inhibit glycolysis catalyzed by VX2 tumor slices provided it is used at concentrations of glucose much higher than those normally found in the blood, and provided it is prior incubated with the tumor slices in the absence of glucose. Here, it can be seen that half maximal inhibition of glycolysis requires about 15 mM 2DOG whereas maximal inhibition (70%) requires almost 50 mM. Results presented in Fig. 3B show that 3BrPA is a more effective inhibitor than 2DOG as it induces half maximal inhibition of the glycolytic activity of the VX2 tumor slices at a concentration of only 2.4 mM and complete inhibition at about 15 mM.

Additional experiments were undertaken to determine whether 3BrPA is also an inhibitor of the mitochondrial hexokinase of the VX2 tumor, as this enzyme is known to be markedly elevated in rapidly growing hepatomas [8–13], and where examined carefully, to be required for maintenance of the high glycolytic rate [9]. Results presented in Fig. 3C show that 5 mM 3BrPA inhibits completely glucose initiation of the hexokinase reaction in a system (see Section 2) containing among other ingredients VX2 tumor mitochondria, ATP, glucose-6-phosphate dehydrogenase, and $NADP^+$. (Although complete inhibition of hexokinase activity is achieved at a concentration of only 5 mM 3BrPA (Fig. 3C),

whereas a concentration near 15 mM is necessary to completely inhibit glycolysis (Fig. 3B), this is likely due to the fact that the former assay was conducted on a cell free extract whereas the latter was conducted on intact tumor tissue). Finally, in addition to these findings, was the very important discovery that 3BrPA (1.2 mM) also completely inhibits mitochondrial respiration (Fig. 3D), i.e. both the basal rate of respiration catalyzed by the respiratory substrate succinate, and the ADP stimulated rate of respiration normally associated with ATP synthesis by oxidative phosphorylation.

3.4. Comparison of the relative capacities of 2DOG and 3BrPA to kill hepatoma cells expressing the high glycolytic/high hexokinase phenotype

The studies described above using the VX2 tumor model for liver cancer would predict that 2DOG and 3BrPA may have the capacity to kill hepatoma cells expressing the high glycolytic/high hexokinase phenotype. For this reason, both agents were tested for their capacity to inhibit the growth of AS-30D hepatoma cells, an established rat cell line known to exhibit a high glycolytic rate and to contain elevated levels of mitochondrial bound hexokinase. Significantly, comparison of control hepatoma cells (Fig. 4A) with those treated 12 h with 20 mM 2DOG (Fig. 4B) or with 5 mM 3BrPA (Fig. 4C) show that both 2DOG and 3BrPA have the capacity to induce cell death, with 3BrPA killing all cells in the population and 2DOG killing about 80%. Cell killing was assessed in these experiments by counting those cells in which trypan blue had entered.

4. Discussion

As indicated earlier, the VX2 tumor (Fig. 1A), although not of hepatic origin, has proven useful as a model for liver cancer particularly in the study of the delivery of anticancer agents via the process known as chemoembolization. Thus, when this tumor is implanted in the rabbit liver it grows rapidly (Fig. 1C,D) and develops a vascularization system similar to that of many human hepatomas. This allows potential anticancer agents to be delivered selectively to the tumor via the hepatic artery [3,4]. However, lack of specific knowledge about the VX2 tumor's metabolism has limited its current utility to the study of drug delivery to liver tumors, leaving its potential usefulness as a model system for also testing novel drugs for arresting the growth of liver tumors open to question.

Significantly, results of studies reported here show that the rabbit VX2 tumor exhibits glucose metabolic properties highly similar to rapidly growing hepatomas. Specifically, the VX2 tumor is shown to exhibit the high glycolytic/high hexokinase phenotype (Fig. 2A–C), and the latter enzyme is shown to be markedly elevated (Fig. 2C), to localize predominantly in the mitochondrial fraction (Fig. 2D), and to exhibit a low Km for glucose (Fig. 2E,F). As emphasized in earlier work, this phenotype, which is characteristic of many rapidly growing hepatomas [7–13], provides a mechanism for the rapid production of glucose-6-phosphate, which not only serves to fuel the glycolytic reaction pathway for the production of ATP, but serves also as a precursor for many biosynthetic processes essential for rapid tumor growth [16–18]. Noteworthy also is that the findings reported here are consistent with the earlier study of Oya et al. [19]showing that the uptake of a 2DOG analog and the activities of several key glycolytic enzymes are elevated in the VX2 tumor relative to liver tissue.

The above studies, suggested that the VX2 tumor growing in rabbit liver could be used not only to study the delivery of anticancer agents to the site of the tumor as previously [3,4], but could be used also to test the effectiveness of tumor glycolytic inhibitors as potential anti-hepatoma agents. For this reason, we carried out a limited screen for such inhibitors. Of the nine compounds tested in this study, two were found to be effective inhibitors of both glycolysis and the growth of hepatoma cells in culture. One of these, i.e., 2DOG, is a known glycolytic inhibitor which is not further metabolized when phophorylated by hexokinase [15], while the other, 3BrPA, was shown in this study to be both an inhibitor of tumor glycolysis (Fig. 3B) acting directly at the level of the hexokinase step (Fig. 3C), and also an inhibitor of the mitochondrial ATP synthetic machinery (Fig. 3D). Of these two inhibitors, 3BrPA was found also to completely kill hepatoma cells in tissue culture following a 12 h incubation period (Fig. 4C), whereas 2DOG, although effective in killing 80% of the cells in the same period of time (Fig. 4B), required a much higher concentration. Future studies will focus on

delivering these and related agents via chemoembolization procedures to the VX2 tumor growing in the rabbit liver, and assessing their capacity to inhibit tumor growth.

Acknowledgements

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EXHIBIT B

[CANCER RESEARCH 62, 3909–3913, July 15, 2002]

Advances in Brief

Novel Therapy for Liver Cancer: Direct Intraarterial Injection of a Potent Inhibitor of ATP Production[1]

Jean-Francois H. Geschwind,[2] Young H. Ko, Michael S. Torbenson, Carolyn Magee, and Peter L. Pedersen[3]

Division of Cardiovascular and Interventional Radiology, The Russell H. Morgan Department of Radiology [J-F. H. G., C. M.], Department of Biological Chemistry [Y. H. K., P. L. P.], and Department of Pathology [M. S. T.], The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2185

Abstract

Most types of cancer are difficult to eradicate and some, like liver carcinomas, are almost always fatal. Significantly, we report here that direct intraarterial delivery of 3-bromopyruvate (3-BrPA), a potent inhibitor of cell ATP production, to liver-implanted rabbit tumors, inflicts a rapid, lethal blow to most cancer cells therein. Moreover, systemic delivery of 3-BrPA suppresses "metastatic" tumors that arise in the lungs. In both cases, there is no apparent harm to other organs or to the animals. Thus, intraarterial delivery of agents like 3-BrPA directly to the site of the primary tumor, followed by systemic delivery only when necessary, may represent a powerful new strategy for arresting the growth of liver and other cancers while minimizing toxic side effects.

Introduction

Liver cancer, in particular hepatocellular carcinoma (hepatoma), is one of the most common fatal cancers in the world (1, 2) and soon may reach epidemic levels because of increased viral-induced hepatitis (3). Among its numerous victims are not only those with primary tumors that develop directly in the liver but those with secondary tumors that frequently arise in this critical metabolic organ as a result of metastasis from other tissues, *e.g.*, the colon (4). Unfortunately, traditional treatment options (5–8) are limited by poor response rates, severe toxicities, and high recurrence rates resulting in a mean survival time of ~6 months. To circumvent these multiple shortcomings, we developed a novel strategy based on knowledge that liver tumors, in contrast to liver tissue, are fed primarily by arterial blood (5, 9), and that the inhibition of ATP production in any cell type quickly induces cell death (10). Specifically, this new strategy involves direct intraarterial delivery to liver tumors of the compound 3-BrPA[4] (Fig. 1, *A* and *B*), a strong alkylating agent (11, 12) that abolishes cell ATP production via the inhibition of both glycolysis and oxidative phosphorylation (Ref. 12; Fig. 1*C*). As described here, we have now demonstrated, using a rabbit model, that this unique approach shows promise as a rapid effective therapy for liver cancer.

Materials and Methods

Tumor Implantation. The rabbit VX2 tumor (5, 12–14) was selected for implantation in the liver because of the similarities of its blood supply to that of human hepatomas. Other attributes of this tumor include rapid tumor growth, development of a sizable tumor that can be readily identified by *X*-ray imaging (fluoroscopy; Ref. 5), and a biochemical phenotype (12) characteristic of advanced stage tumors, *i.e.*, high glycolysis and elevated levels of mitochondrial bound hexokinase (15, 16). In addition, the rabbit is large enough that selective manipulation of catheters in the hepatic artery from the common femoral artery for delivery of agents is possible. Adult New Zealand White rabbits (32 total; Robinson Services, Inc.) weighing 3.5–4.2 kg were used. Studies with these animals were approved by the Johns Hopkins University Animal Care and Use Committee and carried out according to their guidelines. For successful implantation of the VX2 tumor into the liver, the tumor was first grown for 2 weeks on the hind leg of a carrier rabbit. Each carrier rabbit was used to supply tumor cells for implantation into the left lobe of the liver of two separate rabbits. All of the animals, carriers and recipients, were anesthetized with a mixture of acepromazine (2.5 mg/kg) and ketamine hydrochloride (44 mg/kg) administered i.m.; i.v. access was gained via a marginal ear vein, and sodium pentothal was given i.v. to maintain anesthesia. The VX2 tumor was then excised from the carrier rabbit and placed in Hanks' solution. Chunks of the tumor were minced in the same solution. Then, the abdomens of the recipient rabbits were shaved and prepped with betadine, after which a midline subxyphoid incision was made. The anterior surface of the liver was exposed and tumor cells (0.1–0.2 ml) from the minced donor tumor were directly implanted onto the left lobe of the liver using the outer cannula of a 21-gauge angiocatheter. This method allows the growth of a single solitary, well-demarcated tumor in the liver of each recipient rabbit. The abdomen was closed in two layers. Proper aseptic technique was rigorously observed during each implantation. After surgery, animals were returned to their cages, kept warm with blankets, and monitored in the animal laboratory under the direct supervision of a physician or a technician until they recovered from anesthesia. Buprenorphine (0.01 mg) was administered for analgesia when the animals were in pain or showed physical distress. The tumors were allowed to grow for another 14 days, at which time they reached an ellipsoidal shape with dimensions of ~1.5 × 1.8 × 2.5 cm.

Preparation of 3-BrPA Solutions. The solutions of 3-BrPA (Sigma Chemical Co., St. Louis, MO) were prepared in PBS. After adjusting the pH to 7.0 with NaOH the solutions were sterilized via Millipore's Millex GV 0.22 μm filter unit and used immediately. Freshly made solutions were used in all of the studies reported here.

Intraarterial Injection of 3-BrPA. Administration of anesthesia, i.v. access, and sodium pentothal anesthesia were carried out as described above. Transcatheter hepatic artery injection of 3-BrPA was performed under fluoroscopy. The animals were brought to the angiography suite and intubated using a size 3.0-mm endotracheal tube (Mallinkrodt Medical, St. Louis, MO) but not ventilated. Surgical cut-down was performed to gain access into the right common femoral artery, after which a 3 French sheath (Cook Inc., Bloomington, IN) was placed. A specially manufactured 2 French catheter with a tip in the shape of a hockey-stick (JB1 catheter; Cook Inc., Bloomington, IN) was manipulated into the celiac axis, after which a celiac arteriogram was performed to delineate the blood supply to the liver and to confirm the location of the tumor. The tumor could readily be visualized as a region of hypervascular blush located on the left side of the liver near the gastric fundus. The left hepatic artery, which usually provides most of the blood flow to the tumor, was selectively catheterized via the common hepatic artery. When necessary, a steerable guidewire (0.010–0.014 inches Transend wire; Boston Scientific MediTech, Natick, MA) was used to help select the left hepatic

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[2] All of the authors comprising a translational research team contributed equally to this work.

[3] To whom requests for reprints should be addressed, at The Johns Hopkins University School of Medicine, Department of Biological Chemistry, 725 North Wolfe Street, Baltimore, MD 21205-2185. Phone: (410) 955-3827; Fax: (410) 614-1944; E-mail: ppederse@jhmi.edu.

[4] The abbreviation used is: 3-BrPA, 3-bromopyruvic acid.

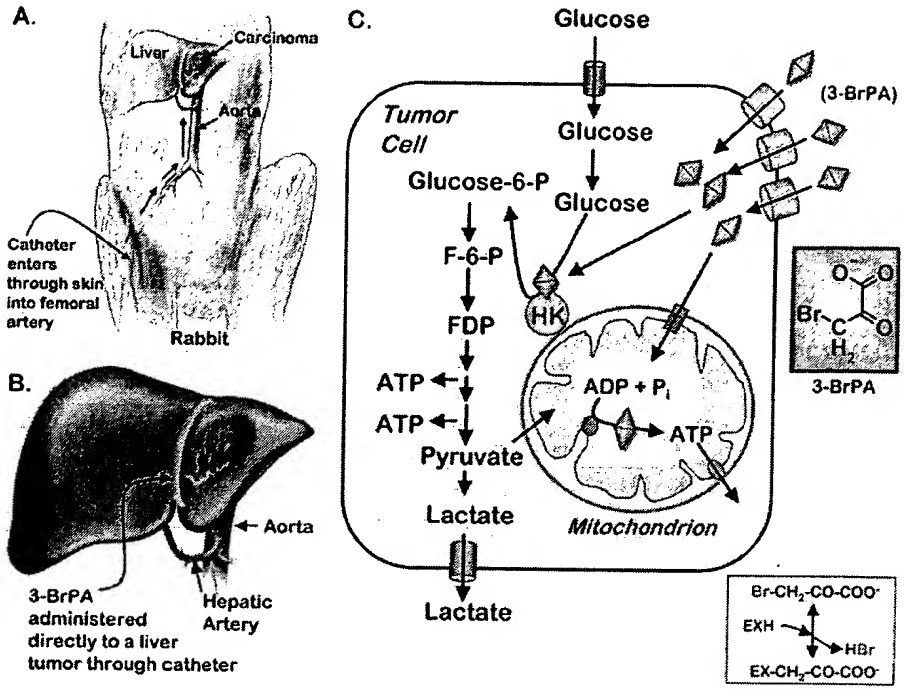

Fig. 1. *A* and *B*, diagrams showing the method used to deliver 3-BrPA intraarterially directly to a liver tumor. *C*, the inhibitory sites of action of 3-BrPA on ATP production, its structure, and its reactivity with enzyme (E) functional groups (*−XH*). Once transported inside a tumor cell, 3-BrPA inhibits ATP production by glycolysis by inhibiting mitochondrially bound hexokinase (12), a metabolic feature of many highly malignant tumors (12, 16), and by inhibiting also ATP production by oxidative phosphorylation (12).

artery. After having adequately positioned the catheter within the left hepatic artery, the 3-BrPA solution was infused directly into the artery. The animals were monitored after the procedure and given analgesics when they showed signs of physical distress.

Embolization. This procedure was performed in a manner similar to the technique described above for 3-BrPA and as described earlier in detail (5). However, instead of using 3-BrPA, a mixture of Ethiodol and embolic material (polyvinyl alcohol; Target Incorporated, Fremont, CA) was injected into the left hepatic artery. The procedure was considered successful when forward flow was no longer demonstrated within the left hepatic artery. In addition, an intense tumor stain was identified in each case, which suggested a successful embolization procedure.

Histopathology. Normal tissues and tumors were fixed in 10% formalin, sliced at 5-mM intervals for gross examination, and then embedded completely in paraffin, after which 4-μm sections were stained with H&E. Tumor viability was estimated by visual inspection and expressed as a percentage of viable tumor area for each slice. The overall percentage of viable tumor in each rabbit was calculated.

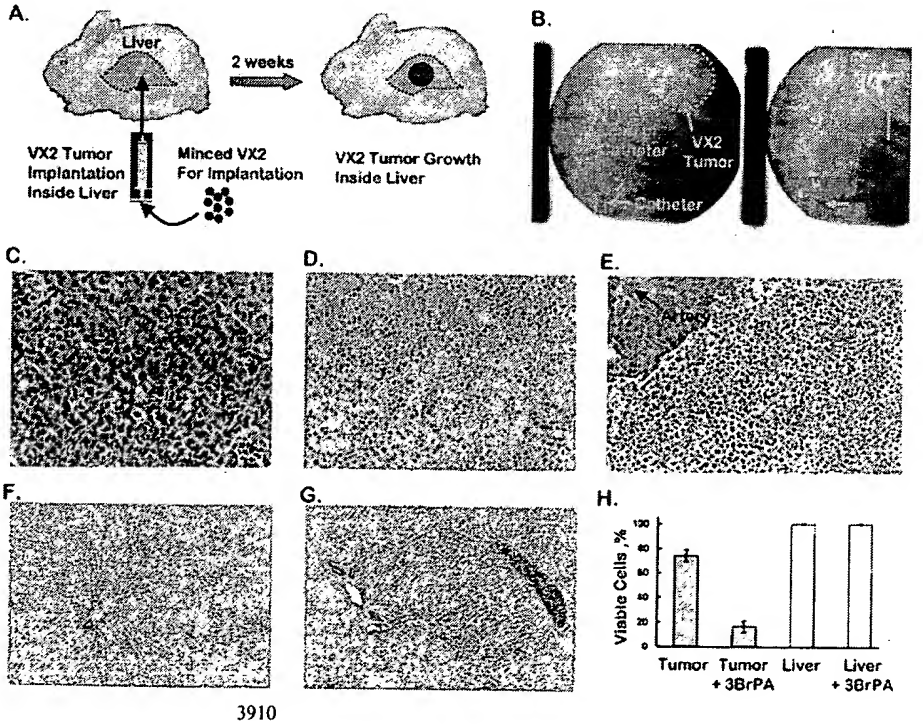

Fig. 2. Experimental setup and effect of intraarterial injection of 3-BrPA on liver tumors. *A*, tumor implantation and growth. *B*, two representative hepatic arteriograms. Each shows the hepatic artery leading into a highly vascularized tumor (*circled*) located within the left lobe. *C*, histological section of a control "untreated" liver implanted tumor isolated 4 days after intraarterial injection of only a saline solution (see "Materials and Methods"). This section, obtained from a region of the tumor located outside of the necrotic tumor core, shows almost all viable cells. (×640). *D*, sections of a liver-implanted tumor isolated 4 days after intraarterial injection of 3-BrPA (see "Materials and Methods"). This section obtained from the same location of the tumor as the control, shows no viable cells. (×640). *E*, sections from a 3-BrPA-treated tumor identical to *D* but showing a region near an artery (*arrow*) where a tiny cluster of cells remains viable. (×640) *F* and *G*, sections from the liver of a control untreated animal and from the liver tissue surrounding an implanted tumor into which 3-BrPA had been injected intraarterially. In both, all of the cells are viable. (×120). *H*, bar graph summarizing the killing efficacy of intraarterial 3-BrPA on liver tumors. Data are plotted as the mean ± SD. For the liver samples, there was no SD because all of the cells tested viable.

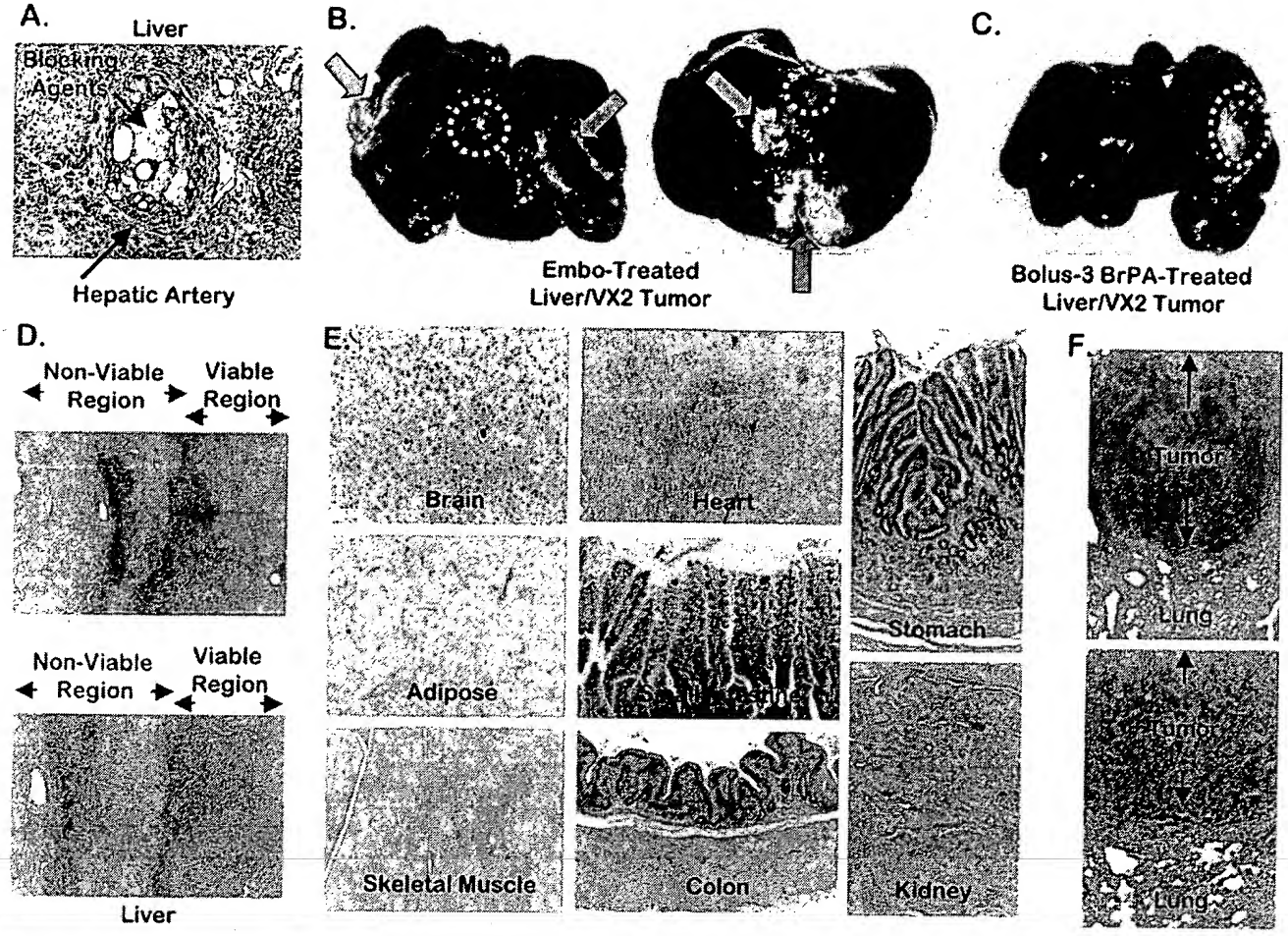

Fig. 3. Evidence for the benefits of intraarterial therapy for liver cancer using 3-BrPA over present therapy using embolization. *A*, view of the left hepatic artery observed microscopically after injection of embolization material (polyvinyl alcohol) and Ethiodol to block blood flow to the liver (5). (×120). *B*, embolized livers harboring VX2-implanted tumors (*circles*). *Arrows*, damage 4 days after embolization. *C*, liver isolated 4 days after its implanted VX2 tumor (*circle*) received a single injection of 3-BrPA. There is no sign of liver damage. *D*, histological sections from those regions of livers shown in *B* that had been affected by embolization. Some tissue has suffered severe damage (nonviable region) and some has remained viable. (×120). *E*, sections of eight tissues from an animal harboring a liver-implanted VX2 tumor treated by intraarterial injection of 3-BrPA. All of the tissues exhibit a normal staining pattern. (×120). *F*, sections derived from the same animal showing metastatic lung tumors. (×120).

Statistical Analysis. The mean fractions of tumor necrosis ± SD were compared using the unpaired Student *t* test for between-group comparisons. Differences were considered statistically significant for $P < 0.05$.

Results and Discussion

Direct Intraarterial Injection of 3-BrPA into Liver-Implanted VX2 Tumors Selectively Inhibits the Viability of Cells Therein without Altering the Viability of Surrounding Liver Tissue. To test our hypothesis that direct intraarterial injection of a potent inhibitor of cell ATP production (3-BrPA) may selectively inhibit the viability of cells within the tumor, we employed the established VX2 tumor model for reasons described under "Materials and Methods." Small chunks of a donor VX2 tumor were minced, surgically implanted in the livers of six rabbits/experiment, and allowed to grow for 14 days (Fig. 2*A*). At this time, the single well-delineated tumor that developed in each liver exhibited a high degree of arterial vascularization because of the onset of angiogenesis. After fasting the animals for 24 h and administering anesthesia, a catheter was carefully inserted into the femoral artery and guided by fluoroscopy into the hepatic artery to a position near the tumor site (Fig. 2*B*). Then, a single bolus injection of 3-BrPA was delivered in ~2 min directly into the artery. Animals treated identically, but not receiving 3-BrPA, served as controls. Optimal results were obtained by delivering 25 ml of 0.5 mM 3-BrPA, waiting 4 days, and then excising and subjecting each tumor and the surrounding liver tissue to histological analysis.

The results obtained from this novel approach proved to be quite dramatic. Compared with control "untreated" tumors, where representative sections (seven slides/tumor) obtained outside the central core region revealed nearly 100% viable cells (Fig. 2*C*), similarly located sections obtained from tumors treated with 3-BrPA (Fig. 2*D*) contained almost all nonviable cells (nearly 100% necrosis). Viable tumor cells were detected only in small areas near arteries feeding the tumors (Fig. 2*E*), and at the tumor periphery, where sinusoidal blood is available. This may reflect more active mitochondria in these oxygen-rich environments that are not completely debilitated at the concentrations of 3-BrPA used. Significantly, no damage occurred to liver tissue surrounding tumors that had been treated with 3-BrPA (Fig. 2 *F* and *G*).

These results, reproduced in a number of experiments, were subjected to statistical evaluation. Tumors untreated with 3-BrPA (controls) contain 74 ± 5% viable cells in the entire population (Fig. 2*H*, *column 1*). The remaining cells, located within the hypoxic tumor core, have already become nonviable, a common feature of rapidly growing solid tumors. Treatment with a single intraarterial injection of

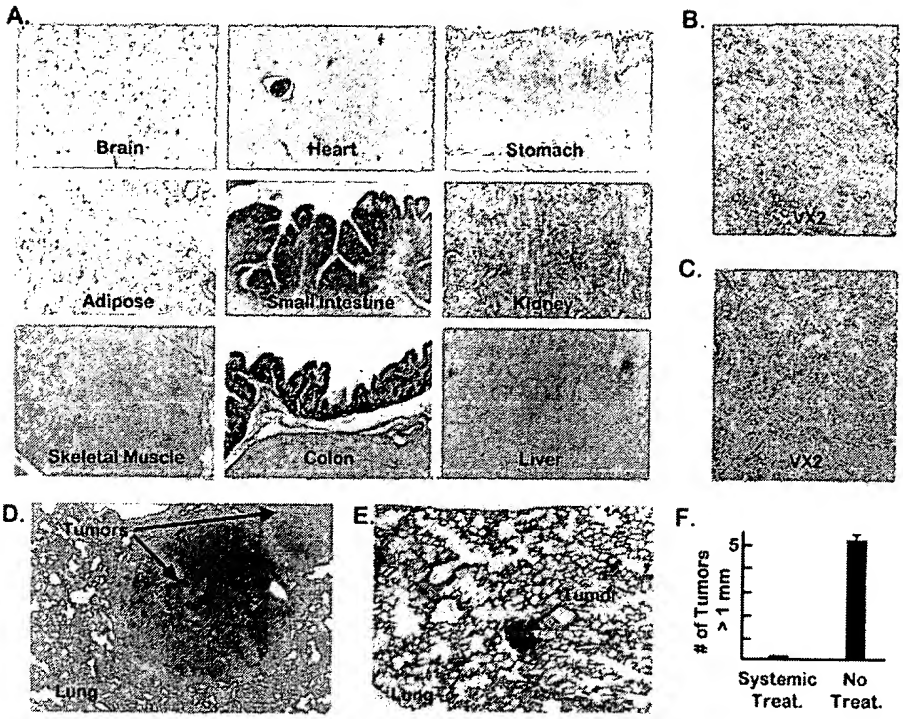

Fig. 4. Effect of systemic delivery of 3-BrPA on animals harboring the liver-implanted VX2 tumor. *A*, histological sections of nine different tissues isolated 4 days after injecting 3-BrPA (25 ml, 0.5 mM) into a marginal ear vein. No damage to these tissues is evident. (×120). *B*, section from a liver-implanted VX2 tumor isolated from a control animal not receiving 3-BrPA. *C*, comparable sample from an animal receiving 3-BrPA systemically. Cells in both appear completely viable. (×120). *D*, section of lung tissue isolated from an animal in which the liver harbored a VX2 tumor after 14 days of growth. *E*, comparable section isolated from the lung of an identical animal 4 days after receiving a systemic injection of 3-BrPA. (×64). The growth of metastatic tumors has been markedly suppressed. *F*, bar graph emphasizing that, of the total number of metastatic lung tumors counted (>27) in comparable histological sections, five were >1 mm in diameter in untreated (*no treat.*) animals harboring a liver-implanted VX2 tumor, and none were >1 mm in identical animals that received 3-BrPA systemically (*systemic treat.*) (Animals evaluated = 4).

3-BrPA decreases the number of viable cells to 16 ± 5% (Fig. 2*H*, *column 2*), thus increasing the total number of nonviable cells in the population to 84 ± 5% ($P < 0.05$). The maximal number of nonviable cells observed in any one experiment was 90%. In sharp contrast, the surrounding liver tissue remained completely viable in all of the cases examined (Fig. 2*H*, *columns 3* and *4*).

In data not presented, the portal veins, sinusoids, and bile ducts remained completely intact, with the only apparent damage occurring occasionally in the peribiliary arteriolar complexes at much higher concentrations of 3-BrPA (5 mM). These and the above findings suggest that most of the 3-BrPA, injected directly into the tumor, remained therein, and if any leakage occurred, most was neutralized by natural reducing agents (*e.g.*, glutathione) present in the surrounding tissue (17, 18).

In Contrast to Direct Intraarterial Injection of 3-BrPA, Conventional Therapy for Advanced-Stage Liver Tumors Using Embolization Results in Significant Damage to Surrounding Liver Tissue. We next inquired how this new strategy compares with the approach, called "embolization" or "chemoembolization," that is currently used to treat advanced stage liver cancer in humans (5, 6, 8, 19, 20). Embolization involves blocking the hepatic artery feeding the tumor with a resin-like material mixed with an oil base (*e.g.*, polyvinyl alcohol in Ethiodol), thus depriving the tumor of its oxygen and nutrient sources. Chemoembolization refers to the same procedure but with the inclusion of one or more anticancer agents. Using the same rabbit model, we found that embolization alone of the hepatic artery (Fig. 3*A*) leading into the VX2 tumor causes such severe damage to the surrounding liver tissue that it is visually evident (Fig. 3*B*). This is in sharp contrast to the normal-appearing liver tissue surrounding VX2 tumors that were not embolized but instead were subjected to direct intraarterial injection of 3-BrPA (Fig. 3*C*). These findings were further substantiated by histological analyses that revealed extensive nonviable liver tissue surrounding tumors treated by embolization (Fig. 3*D*), as opposed to only viable tissue surrounding the tumors treated by intraarterial injection of 3-BrPA (Fig. 2, *F* and *G*).

The Major Tissues of Animals Bearing 3-BrPA-Treated Liver Tumors Show No Apparent Damage, but the Lungs of these Animals and Identical Animals Not Receiving 3-BrPA Show Metastatic Tumors. Despite the promising results obtained in support of direct intraarterial injection of 3-BrPA as a therapy for liver cancer, the possibility still existed that 3-BrPA may be damaging other organs. For this reason, nine major tissues were isolated from animals harboring liver-implanted VX2 tumors 4 days after receiving a single intraarterial injection of 3-BrPA. In no case was there evidence for damage to these tissues (Fig. 3, *E* and *F*). However, the unexpected discovery was made that secondary tumors had developed in the lungs (Fig. 3*F*), a finding observed also in animals bearing liver-implanted tumors that had not been treated with 3-BrPA. Because this was a consistent finding (n = six animals), and because there was no evidence of such tumors in the eight other major tissues examined, these distant lesions are most likely the result of metastatic spread of the VX2 tumor from the liver to the lung.

Systemic Delivery of 3-BrPA Has No Noticeable Effect on the Animals' Health or Behavior and No Effect on Liver-implanted VX2 Tumors, but Does Markedly Suppress the Growth of the Metastatic Lung Nodules. Finally, it was important to examine the effect of 3-BrPA when delivered systemically (*i.e.*, via the general circulation) on both animal toxicity and its capacity to damage liver-implanted tumors. After delivery of 3-BrPA (25 ml, 0.5 mM) via a marginal ear vein, rabbits that had been harboring liver-implanted VX2 tumors for 14 days exhibited normal behavior and, on sacrifice, histological examination of nine major tissues revealed no obvious damage (Fig. 4*A*). Moreover, there was no killing effect on liver-implanted VX2 tumors (Fig. 4 *B* and *C*) as we had observed earlier after direct intraarterial delivery of 3-BrPA (Fig. 2, *C* and *D*), thus adding further support for this targeted approach as a preferred therapy for liver cancer. However, in sharp contrast to the failure of systemic delivery of 3-BrPA to be therapeutic for liver-implanted VX2 tumors (Fig. 4, *B* and *C*), it was found to be therapeutic for secondary tumors that had developed in the lungs. Interestingly,

animals bearing the liver-implanted VX2 tumors developed numerous "metastatic" nodules in their lungs, the largest of which were several mm in diameter (Fig. 4*D*). Most striking in these animals after systemic treatment with 3-BrPA was the finding of only very small tumors (Fig. 4*E*), and the almost complete disappearance of those with a diameter >1 mm (Fig. 4*F*).

In summary, we commenced this study with the objective of testing a novel strategy for the treatment of liver cancer, a strategy that envisioned direct intraarterial injection of 3-BrPA, a potent inhibitor of cell ATP production. We have shown that this strategy is highly effective, reducing in a single injection the total number of viable cells in liver-implanted rabbit tumors to as low as 10% without doing any apparent harm to the animals or their major tissues. As an unexpected extension of our original objective, we have shown also that systemic delivery of 3-BrPA to the same animals bearing the liver-implanted tumors, also does no apparent harm to the animals or their major tissues, but suppresses secondary metastatic tumors that appear in the lungs. Thus, it is possible with a single, carefully selected known chemical agent, and a combination of intraarterial and systemic delivery methods, to inflict extensive damage on both a primary tumor and a secondary metastatic tumor within the same host without doing noticeable harm to the host. Future studies will focus on how the animal's natural defense mechanisms are able to cope with such a reactive alkylating agent as 3-BrPA whereas the liver and lung tumors studied are highly sensitive to this agent.

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